PCRarray 2.0
The second Generation of PCR Array

46400 Benedict Drive, Ste 105
Sterling, VA 20164

(P): 1-866-489-5499
(F): 1-703-637-9863
support@pcrarray2.com
www.pcrarray2.com

qMethyl™ DNA Methylation Detection qPCR System

Simple, Fast, Reliable, No Bisulfite Conversion

(Version 1.2)

For Research Use Only
Not For Use in Diagnostics

1 Receiving, Unpacking and Storing the Products

We recommend that you inspect the package of the products immediately after opening the shipping container and make sure that there are no visible damages to the product. Please contact PCRarray 2.0, Inc. for assistance if there are observable irregularities.

Store the products according to the label on the package. Typically, we recommend that you store qMethyl™ product at -20 °C for storage up to a 12 month period.

Do not open the product container prior use. Water moisture may come in contact with the product and cause adverse effects on the performance.

NOTE: We recommend that you immediately inspect the packages for possible visible damages due to shipment, and store the packages under conditions according to the product label.

2 Table of Contents

1. Receiving, Unpacking and Storing the Products 2
2. Table of Contents 3
3. General Information 4
4. Warnings and Safety Precautions 5
5. Introduction 6
6. Materials Provided 8
7. Additional materials & equipment required 9
8. Consideration before Starting the Experiment 11
9. Protocol 1: 96-well plate PCR array X1 for 1 sample, 44 gene assay 13
10. Protocol 2: 384-well plate PCR array X1 for 4 samples, 44 gene assay 16
11. Protocol 3: 96-well plate PCR array X 2 for 1 sample, 88 gene assay 19
12. Protocol 4: 384-well plate PCR array X1 for 2 samples, 92 gene assay 22
11. Protocol 3: 96-well plate PCR array X 2 for 1 sample, 88 gene assay 19
12. Protocol 4: 384-well plate PCR array X1 for 2 samples, 92 gene assay 22
13. Protocol 5: 384-well plate PCR array X1 for 1 sample, 188 gene assay 25
14. Protocol 6: Multiple sample assay of individual genes with a 96-well or 384-well plate 28
15. Data analysis using ΔCt Methods 32
16. FAQs and Troubleshooting 35

3 General Information

qMethyl™ products are covered by a limited warranty. A copy of the warranty is included with this manual (Appendix, to be added). The operator may be required to store received product under specified conditions as described herein to keep the warranty in effect.

Information in this document is subject to change without notice. PCRarray 2.0, Inc. assumes no responsibility for any errors that may appear in this document. This document is believed to be complete and accurate at the time of publication. In no event shall PCRarray 2.0, Inc. be liable for incidental, special, multiple, or consequential damage in connection with or arising from the use of this product.

qMethyl™ product is designed for research use only and is not intended for use in diagnostic procedures.

Use of the qMethyl™ products conveys no patent rights, expressly or by implication, under any patent or patent application owned or licensed by PCRarray 2.0, Inc., that covers qMethyl™ products. Specifically, but without limitation, no right, immunity, authorization, or license is granted, expressly or by implication, for the usage of qMethyl™ technology.

Limitation of Liability: Except to the extent prohibited by local law, in no event will PCRarray 2.0, Inc. be liable for the direct, indirect, or other damages arising out of the use, inability to use, or the results of using qMethyl™ products. The use of these products is entirely at your own risk.

4 Warnings and Safety Precautions

The following precautions should be followed to minimize the possibility of personal injury and/or damage to property while using qMethyl™ products.

All users should observe good laboratory practices (GLP). Examples of GLP include:

a) Wearing of lab safety equipment, including gloves, eye protection and lab coat when preparing samples.

b) Do not pipette by mouth; do not eat or drink in areas where specimens are being handled.

c) Clean any spill immediately.

d) Disposing of all specimens, controls and materials used in testing as though they contain infectious agents.

5 Introduction

DNA methylation is the enzymatic addition of a methyl group to cytosines in CG dinucleotide DNA sequences (often at CpG-islands, CGI's) without changing primary DNA sequences. Such modifications at gene regulatory regions, in particular promoters, correlate well with the transcriptional state of the cognate gene: hypermethylation represses transcription, while demethylation of previous methylated region reactivates the transcription. It is a fast growing research field in molecular biology, particularly in cancer epigenetic research field.

Our qMethyl gene methylation system is a SYBR green real time PCR based detection system after complete digestion of sample DNA with optimized methylation sensitive restriction enzyme-buffer system. The whole procedure is very simple, fast, and reliable with much less genomic DNA compared to all bisulfite conversion-based methods.

A. The Benefits

1. **Simple & fast:** In two hours, with just 0.5 to 1 µg genomic DNA for one sample, up to 44 target genes can be detected in one run with 96-well PCR plate, or 188 genes with 384-well PCR plate.
2. **Reliable assay design:** All the assays are specifically designed based on established methylated genomic region for known genes;
3. **Complete digestion:** guaranteed complete digestion of sample DNA with the optimized buffer-enzyme system;
4. **Robust quantitative PCR performance**: The PCR primers and cycling parameters are specifically designed for very high GC-rich content; all the PCR reactions are validated;
5. **High sensitivity:** 20 methylated DNA copies can be reliably detected in 5ng genomic DNA in one reaction;
6. **Free, easy to use online Data analysis software:** within one minute, all genes in one run can be analyzed with the exported raw Ct values.

B. The Outline of Simple Three-step Assay

Step 1: DNA Digestion

1) **Mix DNA with the digestion buffer**
2) **Divide the mix equally into two tubes**
3) **Add the enzymes accordingly**
4) **Incubate at 37 °C for 6 hours – overnight**

Step 2: Setup and run the PCR reaction

1) **Mix the two digests with PCR master mix individually , and load to the PCR plate**
2) **Run the Real-time PCR reactions**

Step 3: Data analysis

1) **Collect raw Ct data**
2) **Use analysis template to obtain methylation data**

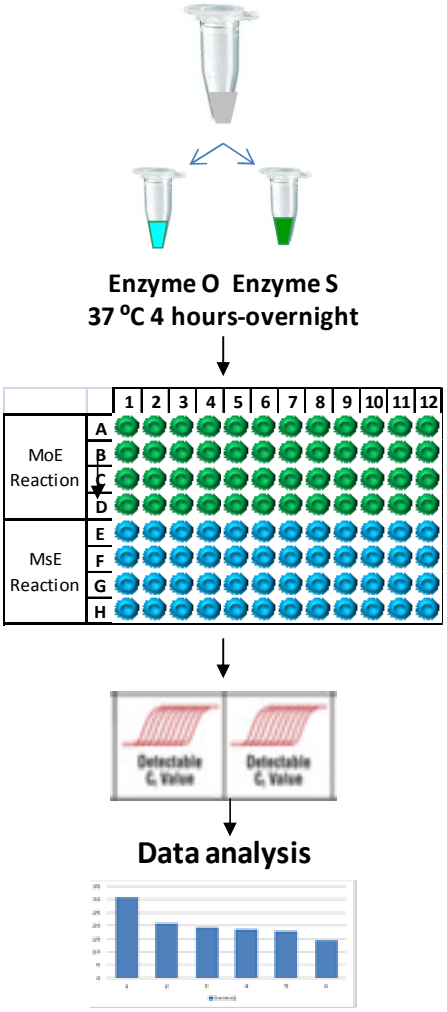

Figure: The simple three-step assay

Step 1: **Mock DNA digestion wit Enzyme O and PCR (MoE):** In the digestions, enzyme blend that do not digest targeted genomic regions is added. The PCR reactions measure the initial input DNA of the target regions.

Step 2: **Methylation sensitive enzyme digestion of DNA with Enzyme S and PCR *(MsE)*:** In the digestion, methylation-sensitive restriction enzyme S is added, so the unmethylated DNA will be cleaved, while the methylated DNA remains intact. The PCR reactions that follow measure the methylated DNA.

Step 3: ***Data analysis***: The hypermethylated and unmethylated DNA fractions in a sample are easily obtained simply by cutting and pasting the raw Ct values into the proper cells of the FREE qMethyl DNA Methylation Data Analysis Excel Template (See the instructions in the excel for detail). It is developed based on ΔCt method with MoE reaction Ct values as initial DNA input signals and those from MsE reaction as hypermethylated signal.

6 Materials Provided

qMethyl array plates are provided based on the following format:

Format	Real-Time Instruments	Plate:
A	All ABI standard blocks (7000, 7300, 7500, 7700, 7900) Bio-Rad iCycler, MyiQ Bio-Rad (MJ Research) Chromo 4 StepOnePlus	96-well
B	ABI 7500 and 7900 FAST 96-well blocks	96-well
C	Stratagene Mx3005p, Mx3000p	96-well
D	Bio-Rad (MJ Research) Opticon and Opticon 2 Stratagene Mx4000	96-well
E	Roche LightCycler 480, 96-well block	96-well
F	ABI 7900 384-well block	384-well
G	Roche LightCycler 480, 384-well block	384-well

NOTE: *The format of a PCR array is indicated by the last digit of the catalog number. Be sure that you have the correct PCR array format for your instrument before starting the experiment.*

For example:

If Cat No.: HMET010-12A is ordered, twelve qMethyl plates of format A will be shipped.

Each PCR array shipment includes the arrays and either twelve (12) optical thin-wall 8-cap strips (Formats A & D) or one (1) optical adhesive film (Formats B, C, E, F & G) per array.

7 Additional Materials & Equipment Required

A. *DNA Isolation Kit*

See our Recommendations in the Protocol Section.

B. *qMethyl Restriction Enzyme Kit EM-03*

MANDATORY for a Complete DNA digestion

C. *qLight SYBR Green qPCR Master Mixes*

MANDATORY for a Complete and Successful Experiment

Note: Be sure to pick the correct one for the instrumentation in your laboratory.

Master mix Type	Cat No	Size	Real-Time PCR cyclers
qLight/ROX SYBR green Master Mix (MR1)	MR1-02	2X 96-well plates/ 200 rxn (2X1.3mL)	All ABI & Stratagene Instrumentation, Eppendorf Mastercycler® ep realplex Instruments with ROX filter set
	MR1-12	12X 96-well plates/ 200 rxn (12X1.3mL)	
	MR1-24	24X 96-well plates/ 200 rxn (24X1.3mL)	
qLight / Fluorescein SYBR Green Master Mix (MF2)	MF2-02	2X 96-well plates/ 200 rxn (2X1.3mL)	BioRad iCylcer®, MyiQ®, and iQ5 Instrumentation
	MF2-12	12X 96-well plates/ 200 rxn (12X1.3mL)	
	MF2-24	24X 96-well plates/ 200 rxn (24X1.3mL)	
qLight SYBR Green Master Mix (MM3 No reference dye)	MM3-02	2X 96-well plates/ 200 rxn (2X1.3mL)	BioRad Opticon, Opticon 2 & Chromo 4, Roche LightCycler® 480 System, Eppendorf Mastercycler® ep realplex Instruments without ROX filter set
	MM3-12	12X 96-well plates/ 200 rxn (12X1.3mL)	
	MM3-24	24X 96-well plates/ 200 rxn (24X1.3mL)	

1). 44-Gene, 96-Well PCR Arrays (Format A, B, C, D, E)
Use the pack sizes of master mix described in the above table.

2). 88-Gene, 96-Well PCR Arrays (Format A, B, C, D, E)
Each a set of 2 array packs requires two (2) of the correct master mix for your instrument (1 X MR-02/MF-02/MM-02).

3). 44-Gene, 384-Well PCR Arrays (Format F, G)
Each 384-well PCR Array 4-pack (Formats F, G) requires four (4) of the correct master mix for your instrument (2 X MR-02/MF-02/MM-02).

4). 92 gene, 384-well PCR arrays (Format F, G)
The two (2), twelve (12), and twenty-four (24) pack sizes (Formats F & G) require a quantity of two (2) of the correct master mixes for your instrument of the size for the corresponding 96-well PCR Array packs (2 X MR-02/MF-02/MM-02 or 2 X MR-12/MF-12/MM-12 or 2 X MR-24/MF-24/MM-24).

5). 188-Gene, 384-Well PCR Arrays (Format F, G)
The two (2), twelve (12), and twenty-four (24) pack sizes (Formats F & G) require a quantity of two (2) of the correct master mixes for your instrument of the size for the corresponding 96-well PCR Array packs (2 X MR-02/MF-02/MM-02 or 2 X MR-12/MF-12/MM-12 or 2 X MR-24/MF-24/MM-24).

D. Additional Equipment & Reagents:

1). For recommendations on specific real-time instrumentation (thermal cyclers with fluorescent detection), see the list of master mixes and plate formats above.
NOTE: The PCR Arrays can only be used in 96-well and 384-well real-time PCR instruments. PCR Arrays can not be used in the Cepheid SmartCycler®, the Roche LightCycler® 2.0, or the Corbett Research Rotorgene.
2). Calibrated Multi-Channel Pipettor
3). RNase / DNase-free pipette tips and tubes
4). RNase / DNase-free 100 µl regular PCR reaction tubes (8- or 12-tube strings)
5). Molecular biology grade ddH_2O (RNase and DNase free)

8 Consideration before Starting the Experiment

Important: Please read through the entire procedure before proceeding to your experiment.

A. Genomic DNA Preparation and Quality Control:

High quality DNA is ESSENTIAL for obtaining accurate results. The most important prerequisite for the DNA methylation detection assay is consistent, high-quality genomic DNA from every experimental sample. It is very helpful to follow the below recommendations.

1). Genomic DNA preparation methods:

In general, popular commercially available DNA purification products are good enough for sample DNA isolation. Here we recommend the followings:

- Cultured Cells: Use 5'Primer's PerfectPure DNA purification kit (Cat # 2302000, Web: www.5Prime.com)
- Tissue Samples: Use Qiagen's QIAamp DNA Mini Kit (manual), or EZ1 DNA Tissue Kit (automatic) (Cat#: 51304 & 953034; Web: www1.qiagen.com)
- Whole blood, bone marrow samples: Qiagen's QIAamp DNA Blood Mini Kit (Manual), or EZ1 DNA Blood 200 µl Kit (automatic) (Cat#:51104, 51204, Web: www1.qiagen.com).
- For Other Biological Samples: Search literatures to find protocols to prepare DNA of high-quality from special biological samples or contact a Technical Support representative.

For best results, all DNA samples should be suspended in DNase-free water, or alternatively in DNase-free 10 mM Tris buffer pH 8.0.

2). Measure DNA concentration and purity by UV spectrophotometer:

Prepare dilutions and measure absorbance in DNase-free 10 mM Tris, pH 8.0 buffer. The spectral properties of nucleic acids are highly dependent on pH.

Concentration by A260 should be greater than 4 µg / ml total DNA.

A260/A280 ratio should be greater than 1.8.

A260/A230 ratio should be greater than 1.7.

3). Avoid contamination of DNA samples:

Whatever DNA isolation methods or commercial kits you may use, DO NOT forget to add RNase to remove RNA. Otherwise, heavy RNA contamination of DNA samples will cause inaccurate DNA quantification and affect DNA digestion efficiency.

4). DNA quality control Assay:

For best results from the PCR array, all DNA samples should also demonstrate consistent high quality. Use our DNA sample quality control kit (Cat#) to assay your DNA sample quality. The assay kit provides control DNA samples and primers for you to test the quality of your DNA samples in parallel. For detailed information, go to our website.

B. Sample DNA amount considerations for an assay

For any single gene assay, the method can yield reliable data with 4 ng genomic DNA of high quality from tissues of single cells types, or from your sample DNA, if 60% of the cells are target cells. As in the cases of any bisulfite conversion-based PCR methods, the result variability will decrease significantly with increased amounts of input DNA. So use larger amounts of input DNA as long as possible when clinical heterogeneous tissue samples are used. For example, when assay are performed to profile gene DNA methylation in cancer cell, DNA amounts should be increased when heavy non-tumor cell contaminations are expected, such as stroma cells, inflammation cells, and blood cells etc.. In general, we recommend the initial input DNA amount per target assay is 10 ng to 30 ng for 96 well blocks, or 2.5 ng to 7 ng /PCR reaction for 384-well block. For an array of 44 gene assays we recommend use 0.6 µg DNA/ sample, and for 188 genes assays with 384-well block, use 1.5 µg DNA.

C. Preparing a clean workspace free of DNA contamination

For reliable results, it is very important to avoid any contamination of the reactions with products of previous PCR experiments. Please follow the recommendations below:

1). Physically separate workspaces for PCR setup from that for post-PCR performance. Before experiment setup, decontaminate PCR workspace, working in a PCR operation hood is highly recommended;

2). Wear gloves throughout the procedure. Use only fresh PCR-grade reagents and lab ware;

3). Prepare dilutions Do not peel the protective film from the PCR array plate until immediate use;

4). Close all tubes containing PCR products once you finish solution transfer.

9 Protocol 1: 96-well plate PCR array X1 for 1 sample, 44 gene assay

Step 1: Setup restriction digestion using qMethyl Restriction Enzyme Kit EM-03:

1) Thaw the 5 X digestion buffer and dissolve the cotton-like precipitates in by vortex briefly. Then, prepare cocktail without the enzymes following the table bellow; we recommend using 1 µg DNA.

Component	Volume
1. ddH_2O	X µl *
2. 5 X ResBuffer	26.5 µl
3. 0.6 - 1.2 µg sample DNA	Y µl *
Total	**120 µl**

* X + Y = 93.5 µl

2) Mix well by vortex, and spin down briefly.

3) Then set up the MoE & MsE digestion reactions in two 200 µl PCR reaction tubes following the table below.

Note: This formula is to ensure that the two reactions contain equal amount of DNA.

Component	MoE digestion	MsE digestion
1. Digestion cocktail	58 µl	58 µl
2. Enzyme MoE	5 µl	0 µl
3. Enzyme MsE	0 µl	5 µl
Total	**63 µl**	**63 µl**

4) Mix thoroughly by inverting the tubes up and down several times.

5) Incubate at 37 °C for 4 hours or overnight in a thermal cycler.

6) Inactivate the enzymes at 65 °C for 20 minutes following the digestion. Store DNA digests at -20 °C till use. Spin down before use.

Step 2: Setup PCR reactions for the two restriction digests:

1) With the two DNA digests, prepare individual PCR cocktails in two 1.5 ml tubes or reservoirs of suitable size following the table below:

Component	MoE mix	MsE mix
1. ddH_2O	490 µl	490 µl
2. PCR master mix	550 µl	550 µl
3. MoE digest	62 µl	-
4. MsE digest	-	62 µl
Total	**1102 µl**	**1102 µl**

2) Mix well, then load the mixes to a signature array plate you have ordered. Aliquot 20 µl of MoE mix to each well from row A to D of the 96-well plate; Aliquot 20 µl of MsE mix to each well from row E to G of the 96-well plate.

Digest		Gene well locations											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
MoE Reaction	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	C	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36
	D	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
MsE Reaction	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	F	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	G	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36
	H	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp

3) *Seal or cap the wells, spin down the plates briefly (2000 rpm for 1 minute).*

4) To run the reactions, use the two-step program shown bellow for all cyclers:

Cycles	Temperature	Duration
1	95 °C	10 minutes[1]
40	98 °C	15 seconds
	75 °C	1 minute[2]
Add melting curve segment	Optional	

Important note:

[1] The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

[2] Detect and record SYBR® Green fluorescence from every well during the annealing/extension step of each cycle.

Step 3: Data analysis with raw Ct values (see below "Section D")

10 Protocol 2: 384-well plate PCR array X1 for 4 samples, 44 gene assay.

Step 1: Setup restriction digestion using qMethyl™ Restriction Enzyme Kit EM-03:

1) Thaw the 5 X digestion buffer and dissolve the cotton-like precipitates by vortex briefly. Then, prepare cocktail without the enzymes following the table bellow. We recommend using 0.5 µg DNA. For each sample

Component	Volume
1. ddH_2O	X µl *
2. 5 X ResBuffer	26.5 µl
3. 0.3~0.8 µg sample DNA	Y µl *
Total	120 µl

* X + Y = 93.5 µl

2) Mix well by vortex, and spin down briefly.

3) Set up four digestion reactions following the table bellow:

Note: This formula is to ensure that the two reactions contain equal amount of DNA.

Component	MoE digestion	MsE digestion
1. Digestion cocktail	58 µl	58 µl
2. Enzyme MoE	5 µl	0µl
3. Enzyme MsE	0 µl	5 µl
Total	63 µl	63 µl

4) Mix thoroughly by inverting the tubes up and down several times.

5) Incubate at 37 °C for 4 hours or overnight in a thermal cycler.

6) Inactivate the enzymes at 65 °C for 20 minutes following the digestion. Store DNA digests at -20 °C till use. Spin down before use.

Step 2: Setup PCR reactions for the two restriction digestion products:

1) In two 1.5 ml tubes, prepare PCR cocktails with the two DNA digests from each sample following the table below:

Components	**MoE Mix**	**MsE Mix**
1. ddH_20	198 µl	198 µl
2. PCR Master mix	260 µl	260 µl
3. MoE digest	62 µl	-
4. MsE digest	-	62 µl
Total	520 µl	520 µl

2) Mix well by vortex, and spin down briefly.

3) Add 10 µl of the PCR mixes for each sample to the proper wells in the 384-well plate following the guidance shown in the figure below:

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Sample 1	MoE	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MoE	B	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
	MsE	C	G01	G02	G01	G02	G01	G02	G01	G02	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MsE	D	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
Sample 2	MoE	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MoE	F	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
	MsE	G	G01	G02	G01	G02	G01	G02	G01	G02	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MsE	H	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
Sample 3	MoE	I	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MoE	J	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
	MsE	K	G01	G02	G01	G02	G01	G02	G01	G02	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MsE	L	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
Sample 4	MoE	M	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MoE	N	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
	MsE	O	G01	G02	G01	G02	G01	G02	G01	G02	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MsE	P	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp

4) Seal the wells, and spin down briefly

5) To run the PCR reactions, use the two-step cycling program shown below for all of PCR cyclers:

Cycles	**Temperature**	**Duration**
1	95 °C	10 minutes[1]
40	98 °C	15 seconds
	75 °C	1 minute[2]
Add melting curve segment	Optional	

Important note:

[1] The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

[2] Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

Step 3: Data analysis: Refer to section D for Data analysis.

11 Protocol 3: 96-well plate PCR array X 2 for 1 sample, 88 gene assay

Step 1: Set up Restriction Digestion using qMethyl™ Restriction Enzyme Kit EM-03

1) Thaw the 5 X digestion buffer and dissolve the cotton-like precipitates in by vortex briefly. Then, prepare cocktail without the enzymes following the table bellow. We recommend using 4 µg DNA.

Component		Volume
1.	ddH2O	X µl *
2.	5 X RM Buffer	42 µl
3.	1.5-3.0 µg Sample DNA	Y µl *
Total		**190 µl**

* X + Y = 148 µl

2) Mix well by vortex, and spin down briefly.

3) Then set up the MoE and MsE digestion reactions in two 200 µl PCR reaction tubesfollowing the table bellow:

Note: This formula is to ensure that the two reactions contain equal amount of DNA.

Component		MoE digestion	MsE digestion
1	Digestion cocktail	90 µl	90 µl
2	Enzyme MoE	10 µl	-
3	Enzyme MsE	-	10 µl
Total		100 µl	100 µl

4) Mix thoroughly by inverting the tubes up and down several times.

5) Incubate at 37 °C for 4 hours or overnight in a thermal cycler.

6) Inactivate the enzymes at 65 °C for 20 minutes following the digestion. Store DNA digests at -20 °C till use. Spin down before use.

Step 2: Setup PCR reactions for the four restriction digests

1) Prepare PCR cocktails: prepare the PCR cocktails with the two DNA digests in two reservoirs as below:

Component	MoE mix	MsE Mix
1. ddH_2O	1000 µl	1000 µl
2. PCR master mix	1100 µl	1100 µl
3. MoE digest	100 µl	-
4. MsE digest	-	100 µl
Total	2200 µl	2200 µl

2) Mix well by vortex, and spin down briefly. Then,

Aliquot 20 µl of MoE mix to each well from rows A to D of the 96-well plate 1 (gene 1 to gene 44) and plate 2 (gene 45 to gene 88);

Aliquot 20 µl of MsE mix to each well from rows E to G of the above two 96-well plates:

Plate 1

Digest		Gene well locations											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
MoE Reaction	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	C	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36
	D	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp
MsE Reaction	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	F	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	G	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36
	H	G37	G38	G39	G40	G41	G42	G43	G44	DC1	DC2	DC3	PCRp

Plate 2

Digest		Gene well locations											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
MoE Reaction	A	G45	G46	G47	G48	G49	G50	G51	G52	G53	G54	G55	G56
	B	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68
	C	G69	G70	G71	G72	G73	G74	G75	G76	G77	G78	G79	G80
	D	G81	G82	G83	G84	G85	G86	G87	G88	DC1	DC2	DC3	PCRp
MsE Reaction	E	G45	G46	G47	G48	G49	G50	G51	G52	G53	G54	G55	G56
	F	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68
	G	G69	G70	G71	G72	G73	G74	G75	G76	G77	G78	G79	G80
	H	G81	G82	G83	G84	G85	G86	G87	G88	DC1	DC2	DC3	PCRp

3) *Seal or cap the wells, Spin down the plates briefly (2000 rpm for 1 minute).*

4) To run one plate first with the two-step program shown bellow for all cyclers. *Keep the remained one in 4 °C and run it immediately after the first one is finished if you have only one machine*:

Cycles	Temperature	Duration
1	95 °C	10 minutes[1]
40	98 °C	15 seconds
	75 °C	1 minute[2]
Add melting curve segment	Optional	

Important note:

[1] The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

[2] Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

Step 3: Data analysis: Refer to section D for Data analysis

12 Protocol 4: 384-well plate PCR array X1 for 2 samples, 92 gene assay

Step 1: Setup restriction digestion using qMethyl™ Restriction Enzyme Kit EM-03

1) Thaw the 5 X digestion buffer and dissolve the cotton-like precipitates in by vortex briefly. Then, prepare cocktail without the enzymes in a *sterilized* 1.5 ml microtube for each sample following the table below. We recommend using 1.5-2.5 µg DNA for each sample.

2)

Component	Volume
1. ddH2O	X µl
2. 5 X RM Buffer	42 µl
3. 1.5-3.0 µg Sample DNA	X µl
Total	190 µl

* X + Y = 148 µl

3) Vortex at high speed briefly, and spin down.

4) Then set up the MoE and MsE digestion reactions in two 200 µl reaction tubes PCR for each sample following the table below:

Note: This formula is to ensure that the two reactions contain equal amount of DNA.

Component	MoE digestion1	MoE digestion 2
1. Digestion cocktail	93 µl	93 µl
2. Enzyme MoE	10 µl	10 µl
3. Enzyme MsE		
Total	103 µl	103 µl

5) Mix well by inverting the tubes up and down several times.

6) Incubate at 37 °C for 4 hours or overnight in any PCR thermal cycler;

7) Inactivate the enzymes at 65 °C for 20 minutes following the digestion. Then store the DNA digests at -20 °C for two years, or -80 °C indefinitely.

Step 2: Setup and run the PCR reactions for the restriction digests

1) Prepare PCR reaction cocktails**:** In two reservoirs, prepare the PCR cocktails with the two DNA digests for each sample as below:

Components	**MoE mix**	**MsE mix**
1. ddH_20	450 µl	450 µl
2. PCR Master mix	550 µl	550 µl
3. MoE digest	100 µl	-
4. MsE digest	-	100 µl
Total	**1100 µl**	**1100 µl**

2) Mix well by shaking gently;

3) Use 12-channel pipette to add 10 µl of MoE cocktail to each well of rows from A to H; then add 10 µl of MsE cocktail similarly to each wells of rows from I to P.

			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Sample 1	MoE	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MoE	B	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48
	MoE	C	G49	G50	G51	G52	G53	G54	G55	G56	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68	G69	G70	G71	G72
	MoE	D	G73	G74	G75	G76	G77	G78	G79	G80	G81	G82	G83	G84	G85	G86	G87	G88	G89	G90	G91	G92	DC1	DC2	DC3	PCRp
	MsE	E	G01	G02	G01	G02	G01	G02	G01	G02	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MsE	F	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48
	MsE	G	G49	G50	G51	G52	G53	G54	G55	G56	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68	G69	G70	G71	G72
	MsE	H	G73	G74	G75	G76	G77	G78	G79	G80	G81	G82	G83	G84	G85	G86	G87	G88	G89	G90	G91	G92	DC1	DC2	DC3	PCRp
Sample 2	MoE	I	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MoE	J	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48
	MoE	K	G49	G50	G51	G52	G53	G54	G55	G56	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68	G69	G70	G71	G72
	MoE	L	G73	G74	G75	G76	G77	G78	G79	G80	G81	G82	G83	G84	G85	G86	G87	G88	G89	G90	G91	G92	DC1	DC2	DC3	PCRp
	MsE	M	G01	G02	G01	G02	G01	G02	G01	G02	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
	MsE	N	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48
	MsE	O	G49	G50	G51	G52	G53	G54	G55	G56	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68	G69	G70	G71	G72
	MsE	P	G73	G74	G75	G76	G77	G78	G79	G80	G81	G82	G83	G84	G85	G86	G87	G88	G89	G90	G91	G92	DC1	DC2	DC3	PCRp

4) *Seal the plate wells, spin down briefly (2000 rpm for 1 minute).*

5) To run the PCR reactions, use the two-step cycling program shown bellow for all of PCR cyclers

Cycles	**Temperature**	**Duration**
1	95 °C	10 minutes[1]
40	98 °C	15 seconds
	75 °C	1 minute[2]
Add melting curve segment	Optional	

Important note:

[1] The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

[2] Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

Step 3: Data analysis: Refer to section D for Data analysis.

13 Protocol 5: 384-well plate PCR array X1 for 1 sample, 188 gene assay

Step 1: Setup restriction digestion using qMethyl™ Restriction Enzyme Kit EM-03

1) Thaw the 5 X digestion buffer and dissolve the cotton-like precipitates in by vortex briefly. Then, prepare cocktail without the enzymes in a *sterilized* 1.5 ml microtube following the table bellow. We recommend using 4.0 µg DNA.

Component		Volume
1	**ddH2O**	X µl *
2	**5 X RM Buffer**	84 µl
3	**3.0-6.0 µg Sample DNA**	Y µl *
Total		380 µl

* X + Y = 93.5 µl

2) Vortex at high speed briefly, and spin down.

3) *Then set up the MoE and MsE digestion reactions, each in two 200 µl PCR reaction tubes following the table bellow:*

Note: This formula is to ensure that the two reactions contain equal amount of DNA.

Component	MoE digestion1	MoE digestion 2	MsE digestion 1	MsE digestion 2
1. Digestion cocktail	90 µl	90 µl	90 µl	90 µl
2. Enzyme MoE	10 µl	10 µl	-	-
3. Enzyme MsE			10 µl	10 µl
Total	100 µl	100 µl	100 µl	100 µl

4) Mix well by inverting the tubes up and down several times.

5) Incubate at 37 °C for 4 hours or overnight in any PCR thermal cycler;

6) Inactivate the enzymes at 65 °C for 20 minutes following the digestion. Then store the DNA digests at -20 °C for two years, or -80 °C indefinitely.

Step 2: Setup PCR reactions for the restriction digests

1) Prepare PCR reaction cocktails**:** In two reservoirs, prepare the PCR cocktails with the two DNA digests as below:

Components	**MoE mix**	**MsE mix**
1. ddH_20	900 µl	900 µl
2. PCR Master mix	1100 µl	1100 µl
3. MoE digest	200 µl	-
4. MsE digest	-	200 µl
Total	**2200 µl**	**2200 µl**

2) Mix well by shaking gently;

3) Use 12-channel pipette to add 10 µl of MoE cocktail to each well of rows from A to H; then add 10 µl of MsE cocktail similarly to each wells of rows from I to P.

		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*
MoE	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
MoE	B	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48
MoE	C	G49	G50	G51	G52	G53	G54	G55	G56	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68	G69	G70	G71	G72
MoE	D	G73	G74	G75	G76	G77	G78	G79	G80	G81	G82	G83	G84	G85	G86	G87	G88	G89	G90	G91	G92	G93	G94	G95	G96
MoE	E	G97	G98	G99	G100	G101	G102	G103	G104	G105	G106	G107	G108	G109	G110	G111	G112	G113	G114	G115	G116	G117	G118	G119	G120
MoE	F	G121	G122	G123	G124	G125	G126	G127	G128	G129	G130	G131	G132	G133	G134	G135	G136	G137	G138	G139	G140	G141	G142	G143	G144
MoE	G	G145	G146	G147	G148	G149	G150	G151	G152	G153	G154	G155	G156	G157	G158	G159	G160	G161	G162	G163	G164	G165	G166	G167	G168
MoE	H	G169	G170	G171	G172	G173	G174	G175	G176	G177	G178	G179	G180	G181	G182	G183	G184	G185	G186	G187	G188	DC1	DC2	DC3	PCRp
MsE	I	G01	G02	G01	G02	G01	G02	G01	G02	G09	G10	G11	G12	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
MsE	J	G25	G26	G27	G28	G29	G30	G31	G32	G33	G34	G35	G36	G37	G38	G39	G40	G41	G42	G43	G44	G45	G46	G47	G48
MsE	K	G49	G50	G51	G52	G53	G54	G55	G56	G57	G58	G59	G60	G61	G62	G63	G64	G65	G66	G67	G68	G69	G70	G71	G72
MsE	L	G73	G74	G75	G76	G77	G78	G79	G80	G81	G82	G83	G84	G85	G86	G87	G88	G89	G90	G91	G92	G93	G94	G95	G96
MsE	M	G97	G98	G99	G100	G101	G102	G103	G104	G105	G106	G107	G108	G109	G110	G111	G112	G113	G114	G115	G116	G117	G118	G119	G120
MsE	N	G121	G122	G123	G124	G125	G126	G127	G128	G129	G130	G131	G132	G133	G134	G135	G136	G137	G138	G139	G140	G141	G142	G143	G144
MsE	O	G145	G146	G147	G148	G149	G150	G151	G152	G153	G154	G155	G156	G157	G158	G159	G160	G161	G162	G163	G164	G165	G166	G167	G168
MsE	P	G169	G170	G171	G172	G173	G174	G175	G176	G177	G178	G179	G180	G181	G182	G183	G184	G185	G186	G187	G188	DC1	DC2	DC3	PCRp

4) *Seal the plate wells, spin down briefly (2000 rpm for 1 minute).*

5) To run the PCR reactions, use the two-step cycling program shown bellow for all of PCR cyclers

Cycles	Temperature	Duration
1	95 °C	10 minutes[1]
40	98 °C	15 seconds
	75 °C	1 minute[2]
Add melting curve segment	Optional	

Important note:

[1] The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

[2] Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

Step 3: Data analysis: Refer to section D for Data analysis.

14 Protocol 6: Multiple sample assay of individual genes with a 96-well plate or 384-well plate

Step 1: Setup restriction digestion using qMethyl™ Restriction Enzyme Kit EM-03

1) Thaw the 5 X digestion buffer and dissolve the cotton-like precipitates in by vortex briefly. Then, prepare cocktail without the enzymes in a *sterilized* 1.5 ml microtube for each sample following the table below. We recommend using 1.5-2.5 µg DNA for each sample.

Component	**Volume**
1. ddH_2O	X µl
2. 5 X ResBuffer	13 µl
3. 0.1~0.2 µg sample DNA	Y µl
Total	60 µl

* X + Y = 47 µl

2) Vortex at high speed briefly, and spin down.

3) Then set up the MoE and MsE digestion reactions in two 200 µl reaction tubes PCR for each sample following the table below:

Note: This formula is to ensure that the two reactions contain equal amount of DNA.

Component	**MoE digestion**	**MsE digestion**
1. Digestion cocktail	29 µl	29 µl
2. Enzyme MoE	2.5 µl	0 µl
3. Enzyme MsE	0 µl	2.5 µl
Total	31.5 µl	31.5 µl

4) Mix well by inverting the tubes up and down several times.

5) Incubate at 37 °C for 4 hours or overnight in a PCR thermal cycler (at volume 30 µl);

6) Inactivate the enzymes at 65 °C for 20 minutes following the digestion. Then store the DNA digests at -20 °C for two years, or -80 °C indefinitely.

Step 2: Setup and run the PCR reactions for the restriction digests:

1) Prepare PCR reaction cocktails**:** In two real-time PCR reaction tubes, a 96-well PCR plate, or a 384-well plate, prepare the PCR cocktails with the two DNA digests for each sample as below:

Components	**MoE mix**	**MsE mix**
1. ddH_20	5 µl	5 µl
2. PCR Master mix	10 µl	10 µl
3. Primer mix (10 µM)	1 µl	1 µl
4. MoE digest	4 µl	-
5. MsE digest	-	4 µl
Total	20 µl	20 µl

2) Mix well by shaking gently

3) *Seal the plate wells, spin down briefly (2000 rpm for 1 minute).*

4) To run the PCR reactions, use the two-step cycling program shown bellow for all of PCR cyclers.

5)

Cycles	**Temperature**	**Duration**
1	95 °C	10 minutes[1]
40	98 °C	15 seconds
	75 °C	1 minute[2]
Add melting curve segment	Optional	

Important note:

[1] The 10-minute step at 95 °C is required to activate the HotStart DNA polymerase.

[2] Detect and record SYBR® Green fluorescence from every well during the annealing step of each cycle.

If you assay a gene on multiple samples, set up the PCR reaction as below:

a) For a 96-well block, prepare the PCR cocktail without DNA samples first follow the table below:

	sample X 2	sample X 4	sample X 8	sample 12
1. ddH_20	16 µl	32 µl	64 µl	96 µl
2. PCR Master mix	40 µl	80 µl	160 µl	240 µl
3. PCR primer mix	4 µl	8 µl	16 µl	24 µl
Total	60	160	240	360

For a 384-well plate, prepare the PCR cocktail without DNA samples first follow the table below:

	sample X 2	sample X 4	sample X 8	sample 12
1. ddH_20	8 µl	16 µl	32 µl	48 µl
2. PCR Master mix	20 µl	40 µl	80 µl	120 µl
3. PCR primer mix	2 µl	4 µl	8 µl	12 µl
Total	30 µl	80 µl	120 µl	180 µl

b) For 96-well block, add 15 of the cocktails to each PCR tube or well, then add 5 µl of the MoE digests and MsE digests to each tube for a sample accordingly

For 384-well plate, add 7.5 µl of the cocktails to each well of the plate, then add 2.5 µl the MoE digest and MsE digest for each sample the wells

c) S*eal the plate wells or cap the tubes, spin down briefly (2000 rpm for 1 minute).*

d) To run the PCR reactions, use the two-step cycling program shown bellow for all of PCR cycler

Cycles	**Temperature**	**Duration**
1	95 °C	10 minutes[1]
40	98 °C	15 seconds
	75 °C	1 minute[2]
Add melting curve segment	Optional	

Step 3: Data analysis: Refer to section D for Data analysis.

15 Data analysis using ΔCt Methods

Note: The PCR array analysis template in Excel format can be downloaded from the link at http://www.PCRarray2.com/qMethylPCRArray/Plate.php. Click the "PCR Array Data Analysis" link found in the lower "Product Support" section of the gray right-hand sidebar. It will make the analysis very easy.

1 ***Obtaining threshold cycle values***: After the PCR reaction, obtain threshold cycle (C_t) values following instructions of the used instruments. We recommend manually setting the Baseline and Threshold Value

1.1 To define the Baseline, use the Linear View of the amplification plots and set the instrument to use the readings from cycle number 3 through the cycle which is before the earliest visible amplification. This is often cycle 15.

1.2 To define the Threshold Value, use the Log View of the amplification plots and place it above the background signal but within the lower third of the linear phase of the amplification plot.

2 ***Export the Ct values:*** Export the Ct values to a blank Excel spreadsheet following the instruction for your instruments. Perform data analysis with the Ct values and data analysis templates for your assay from our website, following the instructions in "Instruction" worksheet shown in the data analysis templates.

3 ***Data quality analysis:***

3.1 ***MoE Ct values:*** The MoE Ct values for all genes should be within the range of 23 to 29 if 5-10 ng DNA are used. The amplification efficiencies for all primer pairs are larger than 85%.

3.2 ***MsE*** The Ct values in MsE will be same as those of MoE's or higher than those of MoE's, depending on the methylated DNA copy numner in the samples.

3.3 ***Enzyme digestion efficiency estimation:*** In "QC Data report" worksheet of data analysis template excel workbook, check the DC control for digestion completeness. The ΔCt value between average Ct values of the 3 DC controls and those of the MoE values should be larger than 5

4 ***Result analysis:***

4.1 *Relative copy numbers of unmethylated, hypermethylated DNA:* For each assay, columns DM & UM in the "Results" worksheet show the relative copy numbers of

hypermethylated methylated, unmethylated DNA species accordingly. For detailed information on the calculation of these values, refer to appendixes.

4.2 *The plots of the results:* The plots of the results will appear in the "Data Chart" sheet; two plots will appear: (1) Hypermethylated genes; (2) Unmethylated genes for each sample.

4.3 *Homozygous deletions:* When the Ct values from all the four digests for a gene are equal to or larger than 32, genomic homozygous deletion is highly suggested at the locus, e.g. in cancer cell genomic DNA. To validate such results, checking the PCR products is recommended by gel electrophoresis; no single dominant bands between 75 bp and 125 bp should be observed.

4.4 *The significance of the results—Cutoff value setting:* The cutoff for the number of methylated DNA copies is set up by users to indicate if the sample is positive for the gene methylation. The same principles used in bisulfite-based real-time PCR methods apply here too. You may set your cutoff based on the following principles:

In most cases only hypermethylated promoters cause transcription repression of cognate genes, so the cutoff is usually set at 5-15% hypermethylated DNA, i.e. at or above that level it is consider that methylation is positive for the genes. The stringency is dependent on the extent of non-target cell contamination. This is similar to using bisulfite-based PCR methods. experimental design.

16 FAQs and Troubleshooting

1. **Is the technology reliable for heterogenous tissue samples?**
   This technology is highly desirable for quantitative detection of heterogeneously methylated DNA populations; therefore it is suitable for analysis of tissue sample consisting of mixed cell types. This is attributed to
   (1) the high specificity, sensitivity & amplification efficiency of PCR reaction guaranteed by the specifically primer design criteria and reaction parameters;
   (2) four selective digestion of DNA samples that include unique DNA methylation dependent restriction enzyme to eliminate background;
   (3) comparable PCR results from the selective digests;
   (4) specific and complete restriction digestion. ***To get the details, go through the answers to questions 2-5.***

2. **How can it be guaranteed that the restriction digestion of the sample DNA be complete?**
   This is achieved by over digestion of DNA samples using longer time and 20 fold higher amount of the DNA methylation-sensitive enzyme A; unspecific digestion is negligible if there is any. Ten cycle differences between MoE and MsE digest Ct values for 100% unmethylated DNA target, it means 0.01% DNA are not digested, or only 1.5 copies of DNA molecules remain undigested in the target DNA region when 5 ng is used. (A copy of monoploidy human genomic DNA is about 3.3333 pg).

3. **How reliable are the results from the qMethyl™ qPCR assay?**
   The reliability of the technology is guaranteed by the following design principles:
   (1) The guaranteed complete restriction digestion: This is achieved by over digestion of DNA samples using longer time and 20 fold higher amount of the DNA methylation-sensitive enzyme A; unspecific digestion is negligible if there is any. Also refer to the answer to question 2.
   (2) Comparable results from the two digests: For any given target sequences, same primer pairs are used to amplify the two differential digests, i.e. the PCR results are generated from same target DNA sequences and same primers, the only difference is the intact DNA copy number left in the two digests.

   This is a great advantage over bisulfite modification-based PCR methods. For a given target with bisulfite methods, two different primer pairs are used to amplify methylated and unmethylated templates from bisulfite conversion. This will cause biased results due to different amplification efficiency of the two primer pairs. In vitro methylated reference DNA and normalization reaction (such as the one used in MethyLight real-time PCR) has to be used to correct this problem, but it still requires that all the primer pairs show similar amplification efficiency. This is a painful challenge that is achieved only after trial and error optimizations. Further more inclusion of

reference DNA also introduces artifacts caused by in consistent incomplete in vitro methylation of the DNA in addition to extra cost

(3) Better sensitivity: The better sensitivity comes from the facts that the same pair primer amplify all the left DNA in the two digests, not affected by the complex configuration of the methylation patterns (A locus consisting of 24 CpG sites contains 16,777,216 (2^{24}) possible different methylation configurations). Making primer and probe sets capable of monitoring all of them, which may be also biological or pathological relevant, would seem to be impossible. Thus, for MSP-based PCR methods, the primers pick up only small fraction of converted templates whose methylation configurations are complementary to designed primers, so the sensitivity is greatly reduced if the CpG site methylation of target DNA sequences is not 100%.

(4) Guaranteed high specificity and amplification efficiency for GC-rich sequences: We have successfully achieved this with our validated primer design criteria and computer algorithm, the great master mix and the universal PCR reaction parameters. Primers for unconverted DNA are much easier to be designed than those for MSP using our unique primer design software for the high GC-rich sequences. Usually, MSP primers with high amplification efficiency and specificity can not be obtained before trial and error optimization of PCR reactions.

4. Why hypermethylated DNA copies can be reliably detected quantitatively with this technology?

The hypermethylated DNA copies are determined using DNA digested with methylation-sensitive restriction enzyme. The enzyme's recognition sequence contains a CpG dinucleotide. When it is get methylated, the cut will be blocked. The recognition sequence occurs in over 98% CpG islands located in gene promoters. In the amplicon design, as many as possible such sites are included, and only a small fraction of the amplicons contain one such restriction site. Only when the amplicon sequences are densely methylated, can all these sites also get methylated and block cutting the amplicons. Thus after Enzyme A digestion, only densely methylated amplicon sequences are left intact and detected.

Such design is representational, based on the fact that in most cases only hypermethylated promoters are relevant to silencing gene transcription. In the design only one or a few CpG sites within a DNA methylation target sequence are analyzed, and their methylation status are used as reporter signal if target regions are densely methylated or not; it is widely used, such as in restriction digestion-based methods including Southern blot, MSRE and restriction landmark genomic screening (RLGS), as well as bisulfite conversion-based methods, such as methylation-specific PCR (MSP), TaqMan-based MethyLight, MS-SNuPE, GoldenGate DNA Methylation BeadArray, Infinium DNA Methylation BeadChip and COBRA etc..

5. What cause incomplete restriction enzyme digestion?

Incomplete digestion will affect the sensitivity significantly. Complete digestion of DNA sample are guaranteed with EpiQ Restriction Digestion Kit and the protocol (refer to the answer to question 2). The most common reasons are DNA samples of poor quality. Sometime poor DNA quality is encountered when prepared from primary tissues and sometime it is difficult to get DNA samples of

high quality from some organ tissues, such as adult lungs from heavy smoking individuals. Several common reasons are:

(1) Low enzyme activity: The restriction enzymes used are expired much lower amount of the enzymes is used. McrBC will be expired after 6 months, while HhaI will expire after two years. Before use, always check the label of the enzyme microtubes for the expiration date.
(2) DNA samples have heavy RNA contamination: This will inhibit DNA digestion with restriction enzymes and will also cause significant inaccuracy in DNA quantification. Be sure to include any RNase treatment steps in recommended DNA isolation procedures. See Page 12.
(3) Too much DNA is used due to inaccurate quantification or miscalculation. Use right methods and instruments to quantify DNA samples.
(4) Impure DNA: when DNAs are prepared from organ tissues that are difficult for DNA isolation, protein and/or polysaccharide contamination may occur and will inhibit restriction enzyme activity significantly. Use recommended DNA isolation kits (refer to Section IV Part A for genomic DNA preparation)
(5) Incorrect incubation (refer to Section IV Part B).
(6) Organic reagents, such as chloroform, phenol, isopropanol are used in preparation of DNA, and the reagents are not completely removed, etc. Whenever possible, avoid using these enzyme activity-inhibiting organic solvents for DNA isolation.

6. High Ct values from mock-treated (undigested) DNA products:

(1) The initial denaturation step (95 C for 10 min) is not used to activate the Taq polymorphism
(2) The amounts of DNA used are too low: (1) miscalculation; (2) incorrect quantification of sample DNA; (3) heavy RNA contamination. Check your calculations and use correct methods and instruments to isolate and quantify DNA sample.
(3) DNA is degraded: DNA samples are contaminated by microbes due to improper storage of your DNA samples such as at 4 °C. Always store your DNA samples at -20 °C (more than 2 years), and at -80 °C (indefinitely).
(4) Improper storage of primer-preloaded qPCR array plates at high temperature too long.
(5) Loss of activity of the real-time PCR Master Mix due to improper storage, possibly left at high temperature for too long

7. Will pipetting error affect the PCR Array results?

The passive reference dyes in the PCR master mixes, such as ROX and Fluorescein, and other technologies (such as for Roch Lightcycler 480) are used by the real-time PCR systems to normalize variation from well to well. These normalization design, combined with our specific digestion and PCR reaction setup protocol design, the performance system can tolerate minor volume variations caused by pipetting errors. Even a 20% pipetting error causes only 0.05 cycles of (Ct) change.

7. How can I prevent the evaporation of reaction volume from the wells?

Be sure to carefully and completely seal the PCR Array with the optical thin-wall 8-cap strips or the optical adhesive film before placing it into your thermal cycler.

If you have additional questions, please check our website (www.PCRarray2.com) for a more complete listing of Frequently Asked Questions (FAQs), or call our Technical Support Representatives at 1-888-XXX-XXXX or XXX-XXXX.